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Appendix C

Calmodulin

PRODUCTION OF AN ANTIBODY IN RABBIT AND DEVELOPMENT OF A RADIOIMMUNOASSAY*

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Calmodulin, a heat-stable Ca^{2+}-binding protein (M_r = 16,700) found in all eukaryotes, is a multifunctional modulator, mediating many of the effects of Ca^{2+} in cellular functions. The protein was derivatized with 1-fluoro-2,4-dinitrobenzene (DNB) to give 3 mol of DNB/mol of calmodulin (DNB_3-calmodulin). The dinitrophenylated protein was almost as active as native calmodulin in stimulating bovine brain Ca^{2+}-dependent phosphodiesterase. Incorporation of the dinitrophenyl groups renders calmodulin highly antigenic in the rabbit; native calmodulin is a weak antigen. Rabbits immunized with DNB_3-calmodulin produced specific antibody against both DNB_3-calmodulin and calmodulin. Using the immunized serum, a radioimmunoassay was developed for calmodulin, the sensitivity for DNB_3-calmodulin and calmodulin being approximately 0.2 and 2 pmol, respectively.

Although the sensitivity of the radioimmunoassay for calmodulin is comparable to the enzyme assay of calmodulin with Ca^{2+}-dependent phosphodiesterase, the radioimmunoassay affords the detection of calmodulin on the basis of antigenic determinants, and thus measures calmodulin in terms of polypeptide structure instead of its ability to stimulate an enzyme. Further, the accuracy of the radioimmunoassay is not affected by the presence of a heat-labile inhibitor protein, which affects the enzyme assay to give an apparent underestimation.

Calmodulin,[1] a ubiquitous Ca^{2+}-dependent modulatory protein in all eukaryotes examined, was first discovered some 10 years ago in our laboratory as an activator of cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) (2–5). Recent evidence from many laboratories suggests that this protein is, in fact, a multifunctional modulator, mediating the effects of Ca^{2+} in a variety of cellular reactions and processes. The mode of action of calmodulin with respect to phosphodiesterase has been studied extensively. In the presence of micromolar Ca^{2+}, calmodulin undergoes a conformational change to a more helical structure (6–9), which is the active configuration (10, 11); the latter interacts with the apoenzyme of phosphodiesterase to form the active holoenzyme (12, 13). The sequence of events leading to the stimulation of the enzyme may be outlined below:

$$\underset{}{\mathrm{CaM} + \mathrm{Ca}^{2+} \longrightarrow (\mathrm{CaM}^{*}\cdot\mathrm{Ca})}$$
$$\text{active}$$
$$\underset{\text{less active}}{(E)} + \underset{\text{active}}{(\mathrm{CaM}^{*}\cdot\mathrm{Ca}^{2+})} \longrightarrow \underset{\text{active}}{(E^{*}\cdot\mathrm{CaM}^{*}\cdot\mathrm{Ca}^{2+})}$$

where CaM stands for calmodulin, E and $E^{*}\cdot\mathrm{CaM}^{*}\cdot\mathrm{Ca}^{2+}$, the apo- and holoenzyme of phosphodiesterase, respectively; and the asterisk (*) indicates the active conformation. Implicit in this scheme is the regulatory role of Ca^{2+}, the cellular flux of which is believed to govern the activity of the enzyme (14–16). This mechanism also appears applicable to brain adenylate cyclase (17) as well as erythrocyte Ca^{2+}-ATPase (18).

Calmodulin also regulates skeletal muscle phosphorylase kinase (19), myosin light chain protein kinase (20–23), plant NAD kinase (24), Ca^{2+} transport in erythrocytes (25–29) and sarcoplasmic reticulum (30), phosphorylation of membranes (31, 32), and the disassembly of microtubules (33). The mode of action of calmodulin on these systems has not been established.

Calmodulin is a single polypeptide consisting of 148 amino acids, with a molecular weight of 16,700 and a pI of 4.3 (34). The amino acid sequence of calmodulin from bovine brain (34) has been completed, and it appears virtually identical with the partial sequence of bovine uterus (35) and rat testis (36). This, together with the biological (37–39) as well as immunological (40) cross-reactivity of calmodulin from phylogenetically distant organisms, suggests that the amino acid sequence of the protein has been highly conserved.

Although there have been numerous studies on the isolated protein, very little has been done on the physiological functions of calmodulin at the cellular level. An antibody against calmodulin can be a highly useful tool in physiological studies. As part of our long term interest in the biological roles of calmodulin, we started several years ago to prepare an anti-calmodulin serum in the rabbit. Repeated efforts failed to elicit an antibody against calmodulin, even when injected absorbed to poly(L-lysine) (41), polymerized with ethyl chloroformate to produce an insoluble complex (42), or cross-linked to thyroglobulin (43). In addition, the thyroglobulin-linked calmodulin was used to immunize chickens and frogs. In all cases, little or no antibody was detected that would

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[1] Calmodulin has been proposed as a proper name (1) for the heat-stable Ca^{2+}-binding protein referred to previously as activator, activator protein, phosphodiesterase activating factor, phosphodiesterase activator protein, Ca^{2+}-dependent regulator (CDR), troponin-C-like protein, modulator protein, or Ca^{2+}-dependent modulator protein. To avoid the confusion in the literature, many investigators have agreed to adopt calmodulin as the proper name.

recognize the native calmodulin, although using thyroglobulin-calmodulin as the antigen, an antibody was obtained in the rabbit. The antibody produced was directed against the thyroglobulin moiety rather than calmodulin. The difficulty in raising an anti-calmodulin probably reflects the fact that calmodulin is small and acidic and that it lacks tissue and species specificity, attributes generally characteristic of a weak immunogen.

We now report the production in the rabbit of an antibody specific for calmodulin using dinitrophenylated calmodulin as the antigen. While the incorporation of several dinitrophenyl groups into calmodulin did not significantly affect its biological activity, these groups render the derivatized calmodulin highly antigenic, and effectively evoke the production of an antibody against calmodulin. In addition, we have developed a radioimmunoassay for calmodulin. While this work was in progress, Dedman *et al.* (44) reported the production of an immunoglobulin against native calmodulin in the goat. The availability of an antibody against and a radioimmunoassay for this important regulatory protein will aid future studies on its cellular and subcellular localization, as well as its role in various biological functions.

EXPERIMENTAL PROCEDURES

Chemicals—[^{3}H]cAMP (20 Ci/mmol) and ^{125}I (16 Ci/mmol) were purchased from Schwarz/Mann; [^{3}H]cAMP was purified before use (45). Freund's complete adjuvant was obtained from Miles Laboratories and 1-fluoro-2,4-dinitrobenzene from Pierce Chemical Co. Goat anti-rabbit immunoglobulin Fc serum was a gift from Dr. William Walker, St. Jude Children's Research Hospital; rabbit skeletal muscle troponin and its individual subunits were provided by Dr. Thomas C. Vanaman, Duke University. Heat-labile inhibitor protein of cyclic AMP metabolic enzymes was purified from bovine brain (46). All other reagents were of highest analytical grades.

Preparation and Assay of Native and Dinitrophenylated Calmodulin—Calmodulin was purified to homogeneity from bovine brain by a modification of a previous procedure (11). Briefly, the procedure was identical with the previous protocol through the DEAE-cellulose chromatography. Chromatography on a hydroxylapatite column was used in place of the terminal preparative electrophoresis. Calmodulin from the DEAE-cellulose column was dialyzed overnight against 10 mM sodium phosphate buffer, pH 6.4. After dialysis, the protein was applied to a hydroxylapatite column (18 × 1.5 cm) which had been equilibrated with 10 mM sodium phosphate buffer. The column was washed with 500 ml of the phosphate buffer and eluted with a linear gradient of 1 liter of 10 mM sodium phosphate (pH 6.4) and 1 liter of 0.3 M sodium phosphate (pH 6.4). Calmodulin was eluted as a symmetrical peak which appeared homogeneous by gel electrophoresis in a 10 to 20% acrylamide gradient gel under nondenaturing conditions using the buffer system described by Laemmli (47).

Calmodulin was dinitrophenylated at 22°C; 250 μl of calmodulin (4 mg/ml) was incubated with 25 μl of 0.7 M $NaHCO_3$ and 50 μl of 1-fluoro-2,4-dinitrobenzene (DNB). The reaction mixture was vortexed continuously for 15 or 60 min. The sample was loaded onto a Sephadex G-25 column (14 × 0.7 cm) to separate the dinitrophenylated from the unreacted calmodulin; 1-ml fractions were collected. The amount of DNB[2]/mol of calmodulin was determined by monitoring the absorbance at 365 nm, using a molar extinction coefficient $\epsilon = 1.74 \times 10^4$ for DNB-L-lysine (48). It is assumed that the DNB covalently bound to calmodulin exhibits the same extinction coefficient as DNB-L-lysine. The number of DNB/mol of calmodulin estimated probably depicts the arithmetic mean, rather than the actual value.

Both native and derivatized calmodulin were assayed by their ability to stimulate bovine brain calmodulin-deficient phosphodiesterase (37). The reaction mixture (0.1 ml) contained 40 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 50 μM $CaCl_2$, 8 μg of calmodulin-deficient phosphodiesterase, 2 mM [^{3}H]cAMP, and an appropriate amount of calmodulin.

Immunization of Rabbits—Two male New Zealand white rabbits were immunized with derivatized calmodulin containing 3 mol of DNB/mol of calmodulin (DNB_3-calmodulin). The antigen was prepared by mixing 1 ml of DNB_3-calmodulin (0.84 mg of protein/ml) with 1 ml of Freund's complete adjuvant. The suspension was emulsified by a brief sonication, injected subcutaneously into the animal at four to five sites around the scapula on Days 1, 17, 33, and 64. On Day 72, each rabbit was bled from the marginal vein of the ear. The blood was allowed to clot and the whole serum was stored in small aliquots at −90°C. For radioimmunoassay, the two sera were mixed in a 1:1 volume ratio.

Preparation of Calmodulin-specific Immunoglobulin—Serum (5 ml) from each of the two rabbits was combined, diluted with 10 ml of 0.15 M NaCl, 20 mM sodium phosphate ($NaCl/P_i$) containing 2 mM EGTA, and added to 15 ml of Affi-Gel 10 previously coupled to calmodulin (1, 46). The slurry was mixed gently overnight and poured into a column (2.5 × 10 cm). The column was washed sequentially with 85 ml of $NaCl/P_i$ containing 1 mM EGTA, 125 ml of $NaCl/P_i$ containing 1.0 M NaCl, and finally 42 ml of $NaCl/P_i$, and then eluted with 50 ml of $NaCl/P_i$ containing 4 M NaSCN. Ten-milliliter fractions were collected with the wash, and 5-ml fractions thereafter. An aliquot (10 μl) of each fraction was assayed for binding of ^{125}I-calmodulin according to the procedure described for the radioimmunoassay.

Iodination of Calmodulin—Calmodulin and DNB_3-calmodulin were iodinated according to a modification of the chloramine-T procedure (49). The reaction mixture (75 μl) contained 0.1 M sodium phosphate (pH 7.2), 2 mCi of ^{125}I, 5 μg of calmodulin, and 10 μg of chloramine-T, added sequentially at 22°C. Immediately, 30 μg of sodium metabisulfite and 100 μl of 0.1 M potassium iodide were added to stop the reaction. The solution was made 0.05% in Triton X-100, and the iodinated calmodulin was separated from unreacted ^{125}I on a Sephadex G-25 column (14 × 0.7 cm) which had been equilibrated with $NaCl/P_i$ containing 0.02% NaN_3 and 0.05% Triton X-100.

Radioimmunoassay of Native and Dinitrophenylated Calmodulin—Radioimmunoassay was performed in BDS polymer tubes (12 × 75 mm) (Evergreen Scientific). The reaction mixture (final volume 300 μl) contained $NaCl/P_i$, 0.02% sodium azide, 0.05% Triton X-100, and 3 mM EGTA. In addition, each tube received 50 μl of anti-calmodulin serum (diluted 50-fold in $NaCl/P_i$), or 25 μl of anti-calmodulin serum (diluted 50-fold in $NaCl/P_i$) and 25 μl of nonimmune rabbit serum (diluted 50-fold in $NaCl/P_i$). The total amount of serum in the assay tube was equivalent to 1 μl of undiluted serum. Different amounts of noniodinated calmodulin (1,000 to 10,000 ng) or tissue extract (1,000 to 60,000 ng) were added, and the mixture was incubated for 30 min at 30°C. Calmodulin labeled with ^{125}I (13,000 cpm, approximately 65 pg) was added, and the tube was incubated further for 30 min at 30°C, followed by an additional incubation at 4°C for 24 h with constant shaking. Ten microliters of undiluted goat anti-rabbit IgG serum was added, and the mixture was incubated for another 16 h. The precipitate was collected by centrifugation at 20,000 × *g* for 20 min; the supernatant fluid was carefully removed from the precipitate, and the tube counted in a Nuclear Chicago Gamma Spectrophotometer. The data, expressed as the mean of two determinations, were corrected for nonspecific binding to the tube, to normal rabbit IgG and goat anti-rabbit IgG. The radioactivity contributed by these nonspecific bindings amounted to approximately 2% of the total counts.

Preparation of Brain Extract for Assay of Calmodulin—Bovine brain cerebra were obtained fresh from a local abattoir and stored at −20°C until needed. To prepare an extract for radioimmunoassay, the tissue was thawed and minced into small pieces with scissors. Two volumes of Buffer A (50 mM Tris-HCl, pH 7.8, 3 mM $MgSO_4$, and 1 mM dithiothreitol) containing 1 mM EGTA were added and the tissue was homogenized with a Polytron tissue homogenizer (Brinkman) for 1 min at a setting of 3. The operation was repeated three more times. The homogenate was centrifuged at 27,000 × *g* for 20 min and the resulting supernatant recentrifuged at 100,000 × *g* for 1 h. The 100,000 × *g* supernatant was heated to 95°C for 4½ min, and the denatured proteins were removed by centrifugation at 100,000 × *g* for 1 h. The clear supernatant fluid was dialyzed overnight against $NaCl/P_i$, and an appropriate fraction was assayed for calmodulin.

Protein Determination—Protein was determined according to Lowry *et al.* (50) with a slight modification. The protein was first precipitated by adding 10 volumes of 10% perchloric acid and 1% phosphotungstic acid; the sediment was collected with a low speed centrifugation after incubation for 1 h at 0–4°C.

[2] The abbreviations used are: DNB, 1-fluoro-2,4-dinitrobenzene; $NaCl/P_i$, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; IgG, immunoglobulin G.

RESULTS

Production of Anti-Calmodulin Serum in Rabbits—Preliminary efforts to elicit an antibody directed against calmodulin in rabbits have been unsuccessful. Calmodulin appears to be a poor antigen in rabbits, probably because the protein in small, acidic, and its amino acid sequence highly conserved in widely divergent species. These early efforts involved the use of calmodulin in various states: they include polymerization of calmodulin with ethyl chloroformate (42) to yield an insoluble complex, forming a complex with poly(L-lysine) (41), or chemically coupling to thyroglobulin by the carbodiimide procedure (43). In addition, the thyroglobulin·calmodulin complex was used to immunize chickens and frogs. As mentioned before, all of these attempts elicited little or no production of antibody against calmodulin. These results strongly suggest that both the native and the modified calmodulin were not sufficiently antigenic to evoke an anti-calmodulin response; a corollary is that calmodulin must be made more antigenic by the incorporation of certain immunogenic groups. The dinitrophenyl group is known to be particularly effective in eliciting antibody production (48). We reasoned that the incorporation of an appropriate number of dinitrophenyl groups into calmodulin might render it highly antigenic, and that the animal would not only make antibodies against the dinitrophenyl groups but probably also against some endogenous groups of the protein. This approach proved to be successful.

Calmodulin was derivatized with 1-fluoro-2,4-dinitrobenzene; the nitrophenylated calmodulin appeared yellow. The extent of derivatization was proportional to the incubation time; for example, a reaction time of 15 and 60 min incorporated 3.2 and 5.9 mol of DNB/mol of calmodulin, respectively. Although both calmodulin derivatives retained biological activity (Fig. 1), the efficacy diminished with the extent of derivatization. Half-maximum stimulation of phosphodiesterase was obtained with 16 ng of native calmodulin, 20 ng of DNB_3-calmodulin, and 240 ng of DNB_6-calmodulin. Since the biological activity of DNB_3-calmodulin did not differ significantly from that of the native molecule, the derivative was chosen as the antigen for subsequent immunization of the rabbit.

Two rabbits were immunized with DNB_3-calmodulin emulsified with Freund's complete adjuvant as described under "Experimental Procedures." The immunized rabbits were bled one week following each injection, and the sera were tested for the presence of antibody directed against calmodulin or DNB_3-calmodulin. One week after the first injection, the serum reacted positively with DNB_3-calmodulin but not with calmodulin (Fig. 2). On the next bleeding (Day 72), the serum reacted positively with both proteins; moreover, the serum titer increased with subsequent boostings. While the titer for DNB_3-calmodulin leveled off after Day 72, that for calmodulin continued to increase until Day 140, and decrease gradually thereafter. The serum from the first bleeding probably contained antibody directed against the dinitrophenyl groups, and additional boosting elicited the production of an antibody directed against other groups of the antigen. The serum obtained on Day 72 was found suitable to develop a radioimmunoassay. The serum titer for DNB_3-calmodulin was markedly higher than that for calmodulin throughout the course of immunization. The results in Fig. 2 depict the immunization schedule and time course production of anti-calmodulin in one of the two rabbits; that of the other rabbit (not shown) was qualitatively similar.

The immune serum from the rabbit did not give a positive Ouchterlony test, indicating that the anti-calmodulin is not a precipitating antibody. The goat antibody against calmodulin is also nonprecipitating (44).

Radioimmunoassay of Calmodulin—Calmodulin iodinated according to the chloramine-T procedure yielded a specific activity of 90 μCi/μg, equivalent to 0.8 mol of ^{125}I/mol of calmodulin. Calmodulin has two tyrosines, one is buried while the other is exposed (7); iodination probably takes place primarily on the exposed residue. However, we did not determine whether the buried tyrosine was also iodinated, or to what extent. Richman and Klee (52) have recently reported that iodination of calmodulin, up to 1 mol of iodide/mol of calmodulin, did not significantly affect its ability to stimulate Ca^{2+}-dependent phosphodiesterase.

Fig. 3*A* shows the binding of ^{125}I-calmodulin or ^{125}I-DNB_3-calmodulin to various concentrations of anti-calmodulin serum. Binding of ^{125}I-calmodulin was detected with 0.1 μl of the antiserum, whereas much less antiserum was needed to give a detectable binding of ^{125}I-DNB_3-calmodulin. Indeed, 0.001 μl of antiserum bound 15% of the ^{125}I-DNB_3-calmodulin, indicating that the antiserum has a higher titer for DNB_3-calmodulin than for calmodulin, in agreement with the data shown in Fig. 2.

Fig. 3*B* shows a standard curve for the radioimmunoassay of calmodulin and DNB_3-calmodulin. Binding of ^{125}I-calmodulin to the antiserum was progressively inhibited by increasing amounts of nonlabeled calmodulin. Half-maximum inhi-

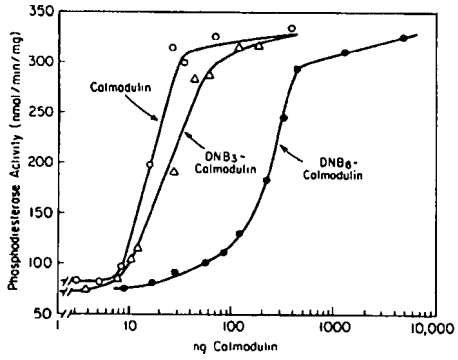

FIG. 1. Effect of dinitrophenylation on the biological activity of calmodulin. The derivative was assayed for its ability to stimulate calmodulin-deficient bovine brain phosphodiesterase (51). The number of DNB/calmodulin is indicated by the *subscripts*.

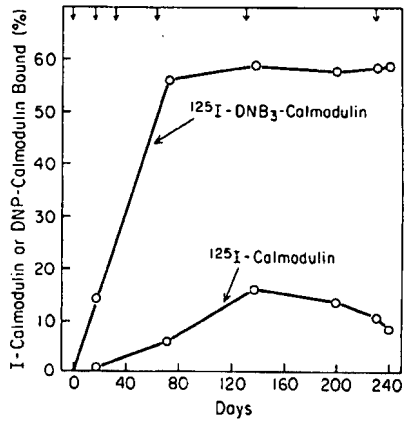

FIG. 2. Immunization schedule and time course appearance of anti-calmodulin in a rabbit serum. The *arrows* indicate the day of injection of DNB_3-calmodulin. The rabbit was bled 1 to 2 weeks after each injection; 0.1 μl of serum was tested for binding of either ^{125}I-calmodulin or ^{125}I-DNB_3-calmodulin, as described under "Experimental Procedures."

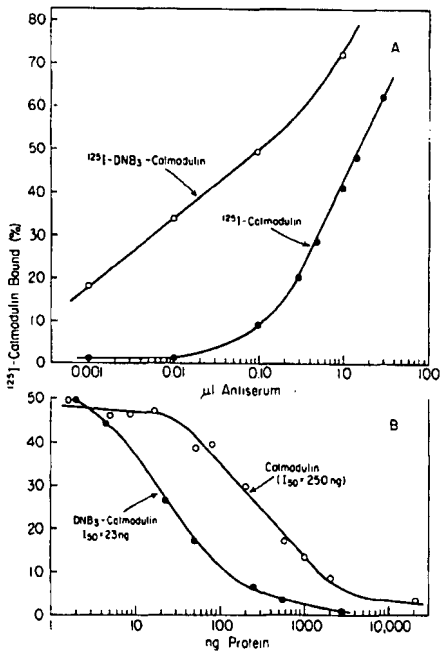

FIG. 3. Radioimmunoassay of calmodulin. *Panel A*, binding of ^{125}I-calmodulin and ^{125}I-DNB$_3$-calmodulin to rabbit anti-calmodulin serum. All assay tubes were adjusted to a constant amount (3 μl) of rabbit serum by the addition of an appropriate amount of nonimmune rabbit serum. ^{125}I-calmodulin or ^{125}I-DNB$_3$-calmodulin bound to the antibody was precipitated with 30 μl of goat anti-rabbit IgG serum. *Panel B*, effect of various concentrations of DNB$_3$-calmodulin and calmodulin on the binding of ^{125}I-calmodulin to the antibody. Each tube contained a constant amount of protein equivalent to 1 μl of rabbit serum. The assay was conducted as described under "Experimental Procedures."

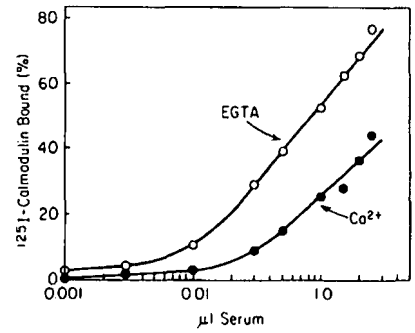

FIG. 4. Effect of Ca^{2+} or EGTA on the binding of ^{125}I-calmodulin to rabbit anti-calmodulin serum. Assays were conducted as described in the legend to Fig. 3 (*Panel A*) in the presence of 1 mM EGTA or 100 μM Ca^{2+}. The sera used here were pooled from two rabbits. The serum from each rabbit was also examined individually; both sera showed a similar decrease in binding ^{125}I-calmodulin in the presence of Ca^{2+}.

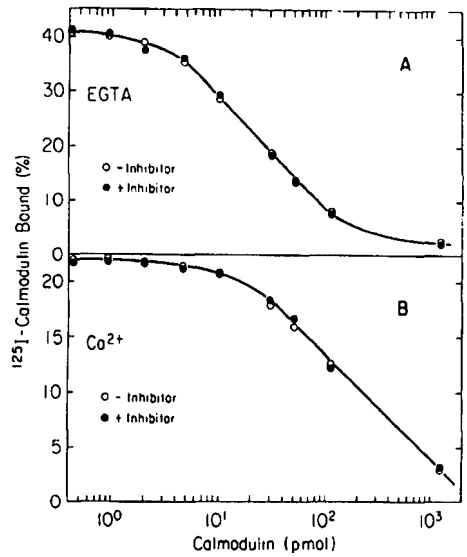

FIG. 5. Radioimmunoassay of calmodulin in the presence or absence of the heat-labile inhibitor protein (46). The radioimmunoassay was conducted as described under "Experimental Procedures" except that the reaction mixture contained either 3 mM EGTA (*Panel A*) or 0.3 mM Ca^{2+} (*Panel B*). When present, the concentration of the inhibitor was 3.8 μg or 48 pmol/assay tube.

bition was obtained with 250 ng of calmodulin or 25 ng of DNB$_3$-calmodulin. The sensitivities of the assay were approximately 30 ng (or 2 pmol) of calmodulin or 3 ng (or 0.2 pmol) of DNB$_3$-calmodulin, while the upper limits of the assay for both molecules were approximately 1000 ng. These data demonstrate that the radioimmunoassay for nitrophenylated calmodulin is 10 times more sensitive. Conceivably, the increased sensitivity by nitrophenylation may be exploited in the future to give a more sensitive radioimmunoassay for calmodulin. Note that the level of calmodulin, which is usually high in many cells and tissues, can be accurately determined with the sensitivity of the radioimmunoassay.

The extent of binding of ^{125}I-calmodulin to the antibody was affected by the Ca^{2+} concentration in the assay mixture (Fig. 4). In the presence of EGTA, the amount of ^{125}I-calmodulin bound was twice that in the presence of Ca^{2+}. Calmodulin is known to undergo a conformational change to a more helical structure in the presence of Ca^{2+} (6–9). The altered conformation in the presence of Ca^{2+} may account for the apparent decrease in avidity of the immunoglobulin for calmodulin. An alternative explanation is that the rabbit serum contains other proteins which bind calmodulin in a Ca^{2+}-dependent manner. In the presence of Ca^{2+}, these components compete with the antibody for binding of ^{125}I-calmodulin, resulting in an apparent decrease of ^{125}I-calmodulin available to the antibody. In the presence of EGTA, this competition could be eliminated.

To test the validity of this notion, the radioimmunoassay of calmodulin was carried out in the presence of a heat-labile calmodulin-binding protein, referred to as an inhibitor protein of cAMP metabolic enzymes (1, 46, 51, 53–55) and erythrocyte Ca^{2+}-ATPase (18). As shown in Fig. 5, the presence of the inhibitor protein (3.8 μg or 48 pmol/tube) did not affect the radioimmunoassay either in the presence of EGTA (*Panel A*) or in Ca^{2+} (*Panel B*). Two possibilities may explain this result. Although the inhibitory protein forms a complex with calmodulin in the presence of Ca^{2+}, the long incubation period involved in the radioimmunoassay could set a new equilibrium in favor of the antigen·antibody complex, nullifying the effect of Ca^{2+}. Alternatively, the antigenic sites of calmodulin are not masked when forming a complex with the inhibitor, and these sites are accessible to the antibody to form an antigen·antibody complex.

In agreement with the results shown in Fig. 4 when the radioimmunoassay was done in the presence of EGTA, the amount of ^{125}I-calmodulin bound to the antibodies was considerably higher; moreover, the titration curve is shifted to the left, giving a greater sensitivity for the assay. The reason for this is not apparent.

Specificity of Anti-Calmodulin—To examine the specificity of the antibody, its cross-reactivity with troponin and its individual subunits troponin-C, troponin-I, and troponin-T, as well as the heat-labile inhibitor protein of cAMP metabolic enzymes from bovine brain (46), was examined. Troponin-C possesses greater than 50% direct sequence homology and

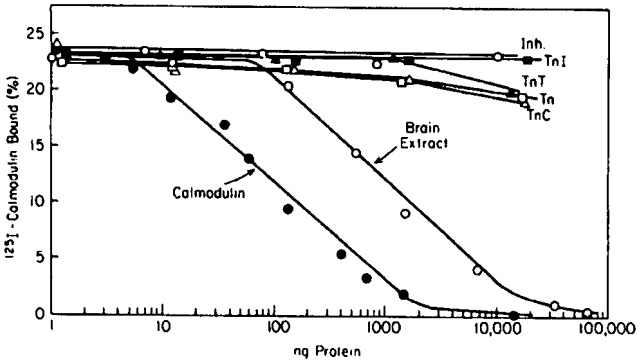

FIG. 6. Effect of various proteins on the binding of ^{125}I-calmodulin to anti-calmodulin serum. Preparation of tissue extract and radioimmunoassay were performed as described under "Experimental Procedures." *Inh.*, inhibitor protein; *TnI*, troponin-I; *TnT*, troponin-T; *Tn*, troponin; *TnC*, troponin-C.

protein profile. In the control experiment using the nonimmune serum, essentially all the protein passed through the column unhindered. Although a trace of protein was eluted with NaSCN, there was no detectable binding by any of the fractions collected from the column. These results demonstrate clearly that the immune, but not the nonimmune sera, contain immunoglobulins with specificity for calmodulin. Indeed, these results are predictable from those obtained by the radioimmunoassay described in Fig. 3.

Comparison of Radioimmunoassay with Enzyme Assay—Since calmodulin is heat-stable, the bulk of extraneous proteins in a brain extract could be conveniently heat-denatured and subsequently removed by centrifugation. To determine the amount of calmodulin that may have been removed along with the heat-denatured proteins, ^{125}I-calmodulin was added to a 100,000 × *g* supernatant fluid of bovine brain extract (as described under "Experimental Procedures"), and the solution was heated in a boiling water bath for 5 min. The denatured proteins were removed by centrifugation, and the amount of radioactivity remaining in the supernatant fluid was determined. Essentially all the radioactivity (90 to 95%) remained in the supernatant fluid, indicating that the denatured proteins did not remove any significant amount of ^{125}I-calmodulin under this condition. The radioactivity remaining in the supernatant after heating could be quantitatively precipitated with 12.5% trichloroacetic acid, suggesting that no isotope was released by heat treatment and that the label in ^{125}I-calmodulin was presumably unaltered.

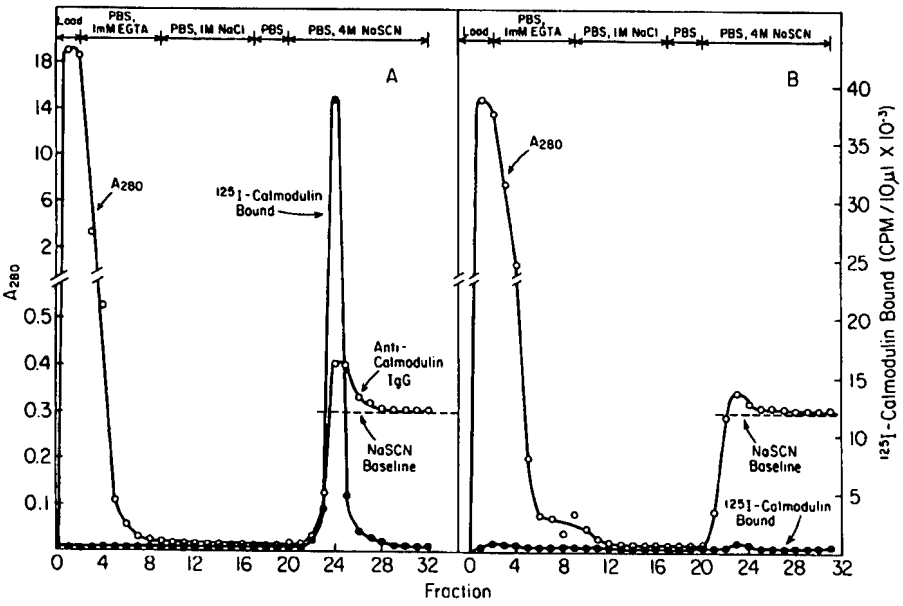

FIG. 7. Preparation of calmodulin-specific immunoglobulin with a calmodulin-agarose affinity column. *Panel A*, immune serum; *Panel B*, nonimmune serum. Chromatography was carried out as described under "Experimental Procedures." NaSCN absorbs at 280 nm which accounts for the elevated baseline at the point of elution by NaSCN. *PBS* ($NaCl/P_i$), phosphate-buffered saline.

greater than 70% conservative sequence homology with calmodulin (34). The inhibitor protein contains an 18,500-dalton subunit which migrates electrophoretically similar to calmodulin in a sodium dodecyl sulfate-acrylamide gel.[3] As shown in Fig. 6, none of these proteins displaced significantly the binding of ^{125}I-calmodulin at the concentrations tested, demonstrating that the antiserum is specific for calmodulin.

Another way of showing that the immune serum from the rabbit contains specific antibodies directed against calmodulin is the use of a calmodulin-agarose affinity column. Fig. 7 depicts the isolation of immunoglobulin from the immune (*Panel A*) and nonimmune (*Panel B*) sera, respectively. With the immune serum, the bulk of the protein passed through the calmodulin-affinity column unimpeded. Subsequent elution with $NaCl/P_i$ containing 1 mM EGTA or 1 M NaCl eluted no significant amount of protein from the column. However, changing the solution to 4 M NaSCN which is known to break the antigen·antibody complex (56) eluted a second, minor protein peak (*Panel A*). An aliquot of the different fractions collected from the column was assayed for its ability to bind ^{125}I-calmodulin. Only the fractions under the second protein peak manifested binding activity, which coincided with the

Fig. 6 also shows the competition by a bovine brain extract for the binding of ^{125}I-calmodulin in the radioimmunoassay. The inhibition curve appears parallel to that of pure calmodulin, indicating the absence of interfering substances in the brain extract. According to the radioimmunoassay, approximately 10% of the protein in the heat-treated supernatant is calmodulin, giving the equivalent of 110 mg of soluble calmodulin/kg of tissue.

The enzyme assay of calmodulin is based on its ability to stimulate a calmodulin-deficient phosphodiesterase, as shown in Fig. 8. According to the enzyme assay, the amount of calmodulin in the extract was 74 mg/kg of tissue. Repeat assay of calmodulin in the extract by the two procedures showed that the enzyme assay consistently gave a lower value.

[3] R. W. Wallace and W. Y. Cheung, unpublished observation.

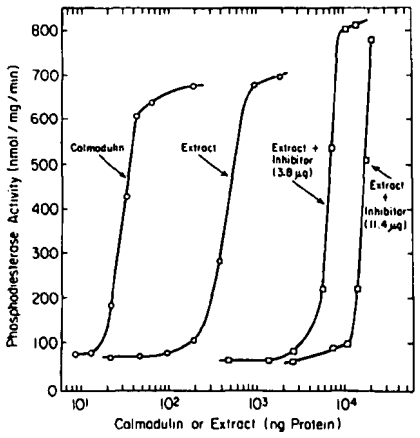

FIG. 8. Enzyme assay of calmodulin by Ca^{2+}-dependent phosphodiesterase. The heat-treated bovine cerebral extract was prepared as described under "Experimental Procedures"; phosphodiesterase activity was determined under standard conditions (51). The *abscissa* refers to the amount of protein contributed by calmodulin or the heat-treated extract. The concentrations of the heat-labile inhibitor protein are indicated in *parentheses*.

Since the enzyme assay was done in the presence of Ca^{2+}, any other calmodulin-binding proteins present in the brain extract would compete with phosphodiesterase for calmodulin, resulting in its apparent underestimation. Calmodulin-binding proteins such as the heat-labile inhibitor protein (46) would be inactivated by the heat treatment. However, bovine brain contains a heat-stable inhibitor protein which binds calmodulin in a Ca^{2+}-dependent manner (57). The presence of this and perhaps other heat-stable, calmodulin-binding proteins could give an apparently lower level of calmodulin obtained by the biological assay.

To demonstrate the interference of the enzyme assay by calmodulin-binding proteins, calmodulin in the brain extract was measured in the presence of two concentrations of the heat-labile inhibitor protein. As shown in Fig. 8, the addition of the inhibitor protein shifted the activity titration curves to the right; the extent of the shift was in direct proportion to the concentration of exogenous inhibitor. In the presence of 3.8 and 11.4 μg of the inhibitor protein, the apparent amount of calmodulin was markedly decreased to 5.8 and 2.2 mg/kg of tissue, respectively. This experiment clearly shows that the presence of the inhibitor gave an apparently lower amount of calmodulin detectable in the brain extract and is in sharp contrast to the radioimmunoassay in which the presence of a comparable concentration of inhibitor (3.8 μg/tube) did not affect the accuracy of the assay (Fig. 5).

Although the sensitivity of the radioimmunoassay is comparable to that of the biological assay, the usable range in the radioimmunoassay is considerably wider (30 to 1000 ng *versus* 10 to 100 ng). Moreover, the radioimmunoassay measures calmodulin on the basis of antigenic determinants, rather than biological activity. In addition, the radioimmunoassay determines calmodulin accurately, even in the presence of potential interfering components, such as the heat-labile inhibitor protein.

DISCUSSION

The dinitrophenylation of calmodulin can be used effectively to provoke the production of an antibody against calmodulin in rabbits. The difficulty in eliciting an antibody response to native calmodulin in the rabbit, frog, and chicken probably reflects the structural similarities between calmodulin from different species. The incorporation of 3 mol of dinitrophenyl residues/mol of calmodulin apparently did not affect appreciably its biological activity. However, the presence of these groups exerts a profound effect on the immunogenicity of calmodulin, as shown by the production of an antibody against DNB_3-calmodulin as early as 1 week following immunization of the rabbit (Fig. 2). The greatly increased antigenicity of calmodulin by the incorporation of dinitrophenyl groups is reminiscent of that of cytochrome *c* (58), which is also a weak immunogen. Upon coupling with one to two dinitrophenyl groups, cytochrome *c* becomes strongly antigenic. The controlled incorporation of dinitrophenyl groups as a means to increase antigenicity may represent a general approach applicable to other proteins that are inherently poor antigens.

Although native calmodulin was antigenically weak in the rabbit, it was effective in the goat. Dedman *et al.* (44) recently demonstrated the production of an antibody against calmodulin in the goat using native calmodulin as the immunogen. The use of rabbit for producing antibody does have practical advantages: the animal is hardy, easily accommodated with a general animal facility, and is less expensive in its upkeep. The goat, on the other hand, provides a much larger volume of serum with each bleeding. This advantage, however, is easily compensated for by using a number of rabbits.

The curves obtained for the differential binding of ^{125}I-calmodulin to the antibody in the presence and absence of Ca^{2+} indicate the presence of conformation-dependent antigenic sites. The difference appears paradoxical because the antibody shows a greater affinity for the less helical conformation that is presumably present in the absence of Ca^{2+}. During the systemic circulation in the host animal, the antigen is exposed to a millimolar concentration of Ca^{2+} in the serum and should assume the more helical conformation; therefore, the antibody should have a higher affinity for calmodulin in the radioimmunoassay carried out in the presence of Ca^{2+}. The results presented in Fig. 4 appear contrary to this argument. One explanation for this paradoxical result, in addition to the ones enumerated earlier, may be that the coupling of dinitrophenyl groups to calmodulin exposes other antigenic sites that are accessible only in the absence of Ca^{2+}.

Although the radioimmunoassay does not offer a greater sensitivity than the enzyme assay, it has the advantage of detecting a wider span of sample concentrations. The presence of numerous Ca^{2+}-dependent, calmodulin-binding proteins in the brain[3] (59) and perhaps in other tissues (60, 61), could cause an underestimation of the tissue level of calmodulin by the enzyme assay. The radioimmunoassay, which measures calmodulin in the presence of EGTA, does not appear susceptible to this interference, and thus would give a more accurate assay. In addition, the radioimmunoassay permits quantitation of calmodulin on the basis of antigenic determinants rather than biological activity. Thus, the method would allow the study of the turnover rate of calmodulin, using an appropriate system, for example, neuroblastoma cells.

Calmodulin and troponin-C have more than 70% conservative sequence homology; moreover, they have many physical chemical properties in common. Yet, the rabbit sera directed against calmodulin does not recognize troponin-C. Although calmodulin effectively substitutes for troponin-C in skeletal muscle myosin ATPase (62), troponin-C has only about 1/600 the effectiveness of calmodulin in stimulating Ca^{2+}-sensitive phosphodiesterase (63). Thus, similarity in physical chemical properties of the two proteins does not necessarily portend their potential biological cross-reactivities.

Although the antibody raised from the immunized rabbits is nonprecipitating, the results presented here demonstrate collectively that the antibody raised in the rabbit is specific

for calmodulin. First, nonimmune serum did not contain any immunoglobulin that binds ^{125}I-calmodulin; in fact, even rabbits that had been injected with the native or one of several forms of modified calmodulin for a prolonged time did not produce any immunoglobulin with specific binding for ^{125}I-calmodulin. Secondly, a calmodulin-specific immunoglobulin was isolated by a calmodulin-agarose affinity column from the immune, but not the nonimmune serum. Thirdly, binding of the antibody for calmodulin is Ca^{2+}-dependent. Calmodulin is known to undergo a conformational change in the presence of Ca^{2+}. Finally, troponin-C, which shares more than 70% of conservative amino acid sequence, also undergoes a Ca^{2+}-dependent conformational change, but is not recognized by the anti-calmodulin serum.

Since the discovery of calmodulin as an activator of cyclic nucleotide phosphodiesterase some 10 years ago (2), extensive studies have been carried out to elucidate its functions at the biochemical level. Increasing evidence from many laboratories suggests that calmodulin is a multifunctional modulatory protein, mediating many of the Ca^{2+} effects in the eukaryotes. Although its ubiquitous distribution in the eukaryotes suggests it may be involved in many of the Ca^{2+}-mediated processes such as cell motility and movement, excitation-contraction and stimulus-secretion coupling, cytoplasmic streaming, chromosome movement, axonal flow, and transmitter release, direct evidence in support of these roles is lacking. The availability of specific antibodies against calmodulin would permit studies that may shed light on these questions. Using immunofluorescent-tagged anti-calmodulin, Welsh *et al.* (64) found that the antibody decorates the mitotic spindle during cell division of fibroblast, indicating a possible role of calmodulin in chromosomal movement. Wood *et al.* (65) noted that in mouse basal ganglia, the antibody labeled the postsynaptic densities and the microtubules associated with postsynaptic dendrites, suggesting that calmodulin may play a postsynaptic role. Cellular and subcellular localization of calmodulin may shed light on its other biological functions.

Of the Ca^{2+}-binding proteins known to act as cellular regulators, calmodulin emerges as a major mediator of Ca^{2+} functions. Future studies of calmodulin should continue to be fruitful at both the biochemical as well as the physiological levels.

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Note Added in Proof—The work of Andersen *et al.* (66), who obtained a precipitating antibody from rabbits, came to our attention after this manuscript was accepted for publication.

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